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Abstract

RNA modification is a post-transcriptional process by which certain nucleotides are altered after their initial incorporation into an RNA chain. Transfer RNAs (tRNAs) is the most heavily modified class of RNA molecules. These modifications expand the chemical and functional diversity of tRNAs and enhance their structural stability. To date, more than 100 modifications have been identified, the majority of which are specific from one domain of life. However, few modifications are extensively present in the three domains of life. Among those, the m^1A nucleotide, which consists in the methylation at position 1 of the adenine aromatic ring, is found in tRNAs and ribosomal RNAs. In tRNAs, the m^1A modification occurs at position 9, 14, 22, 57 and 58. The enzyme TrmK catalyzes the m^1A formation at position 22. Here we report the backbone ^1H, ^{15}N and ^{13}C chemical shift assignments of TrmK from *Bacillus subtilis* obtained by heteronuclear multidimensional NMR spectroscopy as well as its secondary structure in solution as predicted by TALOS+. These assignments of TrmK pave the way for interaction studies with its tRNA substrates.

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Key words: backbone resonance assignment, heteronuclear NMR, unlabeling, methyltransferase, TrmK, tRNA, m1A

1. Biological context

The biosynthesis of transfer RNAs (tRNAs) is a complex process composed of several steps leading to the formation of mature tRNAs with correct structures and functionalities. Apart from the processing of their extremities, the most salient property of the maturation process consists in the post-transcriptional incorporation of a large number of chemical modifications by the so-called modification enzymes. Among the modification enzymes, the methyltransferases (MTases) are the most frequent and diverse (Hori 2014). They catalyze the transfer of a methyl group from a methyl donor, mostly the S-adenosyl-L-methionine (SAM), towards different positions of the nucleotides. The m^1A modification, which consists in the incorporation of a methyl group at position 1 of adenines, occurs on nucleotides 9, 14, 22 and 58 of tRNAs. This modification brings a positive charge on the adenine aromatic ring. We focused our work on the methyltransferase TrmK from *B. subtilis* that catalyzes the methylation of adenine 22 of $tRNA^{Ser}$ and $tRNA^{Tyr}$ (Roovers *et al.* 2008). *B. subtilis* TrmK belongs to

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the COG2384 (Cluster of orthologous groups). The members of this family are found in Gram-negative and Gram-positive bacteria. Their sequences are well-conserved in many bacterial pathogens (*L. Monocytogenes*, *V. Cholerae*, *S. Pneumoniae*...). Since TrmK is essential for cell viability in *S. Pneumoniae* and since no homologues are found in humans, it was proposed to be a good target for the discovery of novel antibiotics (Thanassi *et al.* 2002). We solved the X-ray structure of *B. subtilis* TrmK (Dégut *et al.*, manuscript in preparation) and used NMR chemical shift mapping to get insight on the protein-RNA recognition mode. We report here the backbone chemical shift assignments of TrmK, assignments that were necessary to interpret the NMR chemical shift mapping with its tRNA substrate.

2. Methods and experiments

2.1. *Protein expression and purification*

Recombinant *B. subtilis* TrmK was expressed and purified with a protocol adapted from Roovers *et al.* (Roovers *et al.* 2008). Since wild-type TrmK is prone to aggregation through cysteine oxidation, we produced a protein variant with cysteine to serine mutations (*i.e.* C35S and C152S), which abolished protein aggregation and retained full enzymatic activity (Dégut *et al.*, manuscript in preparation). Mutagenesis was performed by use of the Quickchange site-directed mutagenesis kit (Stratagen). The presence of the desired mutations in *trmK* was checked by sequencing. This variant was overexpressed in BL21(DE3) *E. coli* cells, in rich labeled media or minimum media (see below) supplemented with kanamycin at 30 μg.mL^{-1}. The cells were grown at 37 °C to OD600 ~0.6, cooled down at 18 °C and induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested 24 h after induction by centrifugation and frozen at -80 °C until further use. The frozen cells were suspended in 1/50 of the culture volume of a 50 mM Tris-HEPES buffer pH 8.2 containing 500 mM NaCl, 5% glycerol and 1 mM of phenylmethanesulfonylfluoride (PMSF). The suspension was sonicated and the lysate was centrifuged for 30 min at 45'000 g. The resulting supernatant was loaded to a 5 mL Nickel Sepharose column (HisTrap, GE Healthcare) previously equilibrated with a 50 mM Tris-HCl buffer pH 8.0 containing 500 mM NaCl and 5% glycerol (equilibration buffer). The resin was then washed with 30 mL of buffer and the protein was eluted with a gradient of the equilibration buffer supplemented with 500 mM imidazole pH 8.0. The N-terminal His_6-tag of TrmK was removed by thrombin cleavage (25 U thrombin/mg of protein) performed overnight at 4 °C. PMSF at 250 μM, and EDTA at 1 mM were then added to the protein sample. The sample was concentrated with Amicon 10'000 MWCO (Millipore) and injected on a size exclusion chromatography column (Superdex-75 26/60, GE Healthcare) equilibrated with a 50 mM sodium phosphate buffer pH 7.0 containing 500 mM NaCl and 2% glycerol.

Doubly labeled (^{15}N/^{13}C) and triply labeled (^{2}H/^{15}N/^{13}C) TrmK samples were obtained by growing the cells in Spectra-9CN and Spectra-9DCN media, respectively (Spectra Stable Isotopes, Inc.). Singly labeled (^{15}N) TrmK samples were obtained by growing the cells in M9 minimum media supplemented with $^{15}NH_4Cl$. To achieve high yield of protein in the fully deuterated medium, a double selection protocol of the strain was performed as previously described (Sivashanmugam *et al.* 2009). Besides, specifically unlabeled samples were produced in M9 minimum media supplemented with $^{15}NH_4Cl$ and with non-labeled histidine, arginine, or lysine at a final concentration of 1 mM, 1 hour before induction (Rasia *et al.* 2012). NMR samples of TrmK at ~0.7 mM were prepared in a 50 mM sodium phosphate buffer pH 7.0, 500 mM NaCl, 2% glycerol, 1 mM EDTA, 250 μM PMSF and 10% D2O. The sample was put in a 3 mm-diameter NMR tube.

2.2. *NMR experiments*

All NMR spectra were recorded at 15 °C on Bruker 600, 800, or 950 MHz spectrometers equipped with cryogenic probes. Backbone assignment was performed using the following standard 3D NMR experiments (Salzmann *et al.* 1998): TROSY-HNCA, TROSY-HNCACB, TROSY-HN(CO)CACB and [^{1}H-^{15}N] NOESY-HSQC. A 3D (H)N(COCA)NH experiment was also measured (Bracken *et al.* 1997). Internal DSS standard was used for direct referencing of the ^{1}H chemical shifts, and indirect referencing of ^{15}N and ^{13}C shifts (Wishart *et al.* 1995). Data processing was carried out with Topspin 3.2 for standard acquisition, and with MddNMR (Orek-

hov and Jaravine 2011) for non-uniform sampling acquisition. Analysis of spectrum and backbone assignment was performed with the Sparky suite (Goddard and Kneller). Table 1 summarizes the NMR experiments used for the assignment.

Table 1
NMR experiment recorded for backbone chemical shift assignment of TrmK

Spectra	Spectrometer	^{2}H	Duration	NUS
HNCA	800 MHz	Y	27 h	Y (65%)
HNCACB	800 MHz	Y	86 h	Y (62%)
HN(CO)CACB	600 MHz	Y	90 h	N
(H)N(COCA)NH	600 MHz	Y	89 h	N
HNCO	950 MHz	Y	24 h	Y (35%)
^{15}N-HSQC-NOESY	950 MHz	N	49 h	N

^{2}H: indicates whether TrmK was deuterated (Y: yes, N: no). Duration: indicates the experimental time in hours. NUS: indicates whether non-uniform sampling was used for the acquisition of data (Y: yes, N: no). The percentage in parenthesis indicates the amount of measured data using sparse sampling compared to conventional acquisitions.

3. Resonance assignments and data deposition

Before tackling the assignment of TrmK NMR signals, we needed to cope with problems of precipitation and proteolysis of TrmK. Indeed, TrmK precipitated during concentrations steps, needed high concentrations of salt (500 mM NaCl) and was subjected to intensive proteolysis within a few days after purification. Numerous conditions were tested using dialysis buttons. They allowed us to test the stability of TrmK in various conditions of buffer and temperature while working at high concentrations of TrmK (15 mg/mL) and low volumes (50 μL). As a result, TrmK does not precipitate at high concentration ($>$ 15 mg/mL) in a phosphate buffer whereas we observed precipitation more rapidly in a Tris-HCl buffer. A high concentration of salt (400 to 500 mM NaCl) limited the precipitation of TrmK and its proteolysis. Anti-proteases (PMSF, Pepstatine A, Leupeptine, EDTA) were tested. PMSF and EDTA revealed to be efficient to stop proteolysis. PMSF was added in the lysis buffer and at different stages of purification and in the NMR tube. Lastly, glycerol was necessary to prevent precipitation when concentrating TrmK. 5% of glycerol was an optimal concentration during purification steps and for storage of the protein. For the NMR studies, 2% of glycerol was used as the best compromise between the stability of the protein and the linewidth of NMR signals.

The protein sample in this study consists in 238 residues after the cleavage of the N-terminal His-tag and contains 5 prolines. NMR assignments were based on 3D heteronuclear NMR experiments performed on ^{2}H/^{15}N/^{13}C-labeled TrmK. Interestingly, the use of the (H)N(COCA)NH experiment (Bracken *et al.* 1997), which correlates each NH group to the nitrogen of the amide group of the following residue in the sequence of the protein, revealed to be very efficient for the achievement and the validation of the assignments. This experiment is not sensitive and is commonly used for unfolded protein. However, with a fully deuterated protein, this experiment turned out to be very helpful in the assignment procedure. Besides, we used several specifically unlabeled samples to confirm the assignment or to provide starting point for the assignment (Fig. 1). For instance, Fig.1 shows the unlabeling of all the lysines of TrmK. In this sample, all the amide groups of TrmK are ^{15}N-labeled except those of lysines. Consequently, the lysines are not observable in the red spectrum of Fig. 1. The superimposition with a reference TROSY spectrum (in black, Fig. 1) allows one to easily localize the peaks originating from lysine amide groups.

Backbone amide ^{1}H-^{15}N resonance assignment of TrmK was achieved for 222 of 233 non-proline residues, corresponding to 96% of completeness (Fig. 2). Amide groups from residues M1, S28, H30, A31, L39, N40, H41, K42, T113, E116 and R117 could not be assigned the majority of which are found in loop regions. In addition, 98% of Cα 98% of Cβ and 92% of CO were assigned. The backbone chemical shift assignments of TrmK were deposited in the BioMagResBank (`http://www.bmrb.wisc.edu`) under accession number 26744.

An analysis of the chemical shifts of HN, N, CO, Cα and Cβ atoms was conducted with the TALOS+ webserver (Shen *et al.* 2009). Fig. 3 compares the prediction of TrmK secondary structure obtained from TALOS+ with the secondary structure observed in the crystal structure of TrmK (Dégut *et al.*, manuscript in preparation). The only light dif-

ferences lie in the prediction from the NMR data of short α-helical turns (2 or three residues) around residue 68 and 165 that are not observed in the crystal structure. Therefore, both data are in very good agreement. The TrmK fold in solution and in the crystal is thus identical. Subsequently, the NMR footprint of binding of the tRNA on TrmK will be mapped on the X-ray structure of TrmK.

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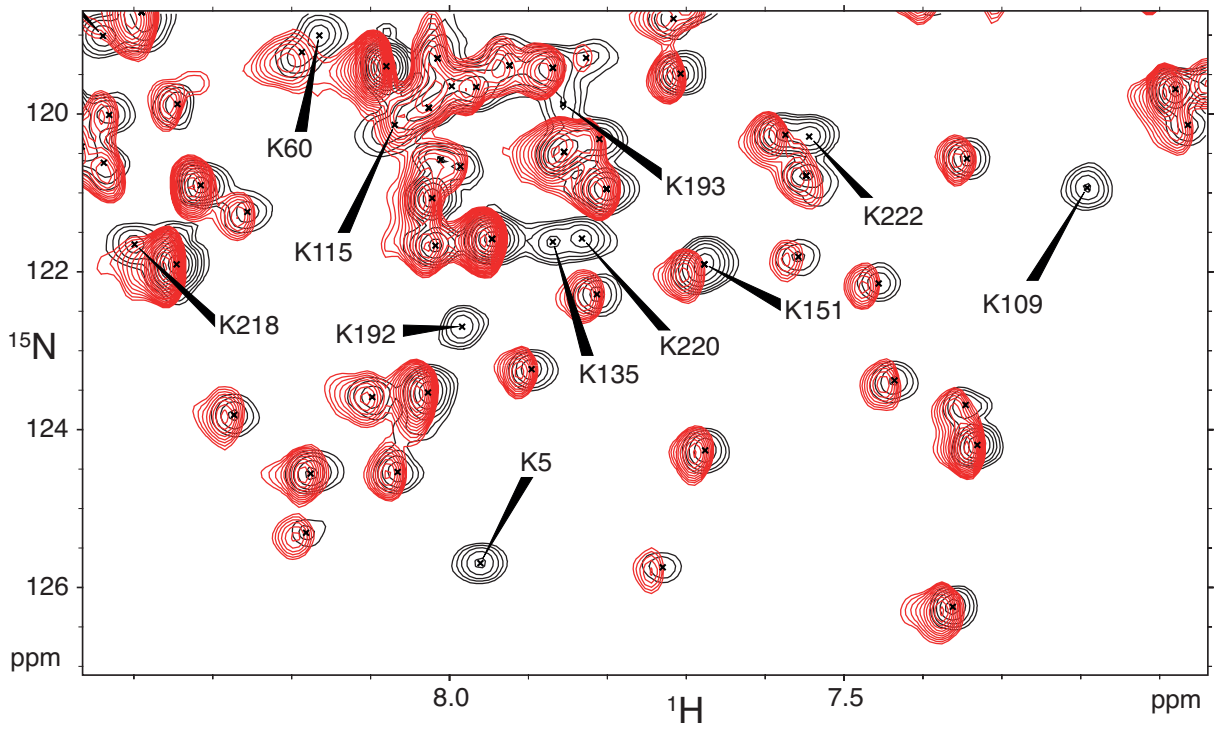

Figure 1. Superimposition of two ^{1}H-^{15}N TROSY spectra of TrmK in black for TrmK uniformly ^{15}N-labeled and in red for TrmK ^{15}N-labeled except on lysines. Lysines are thus not observable on the red spectrum confirming immediately the assignment of amide group for each lysine of the protein.

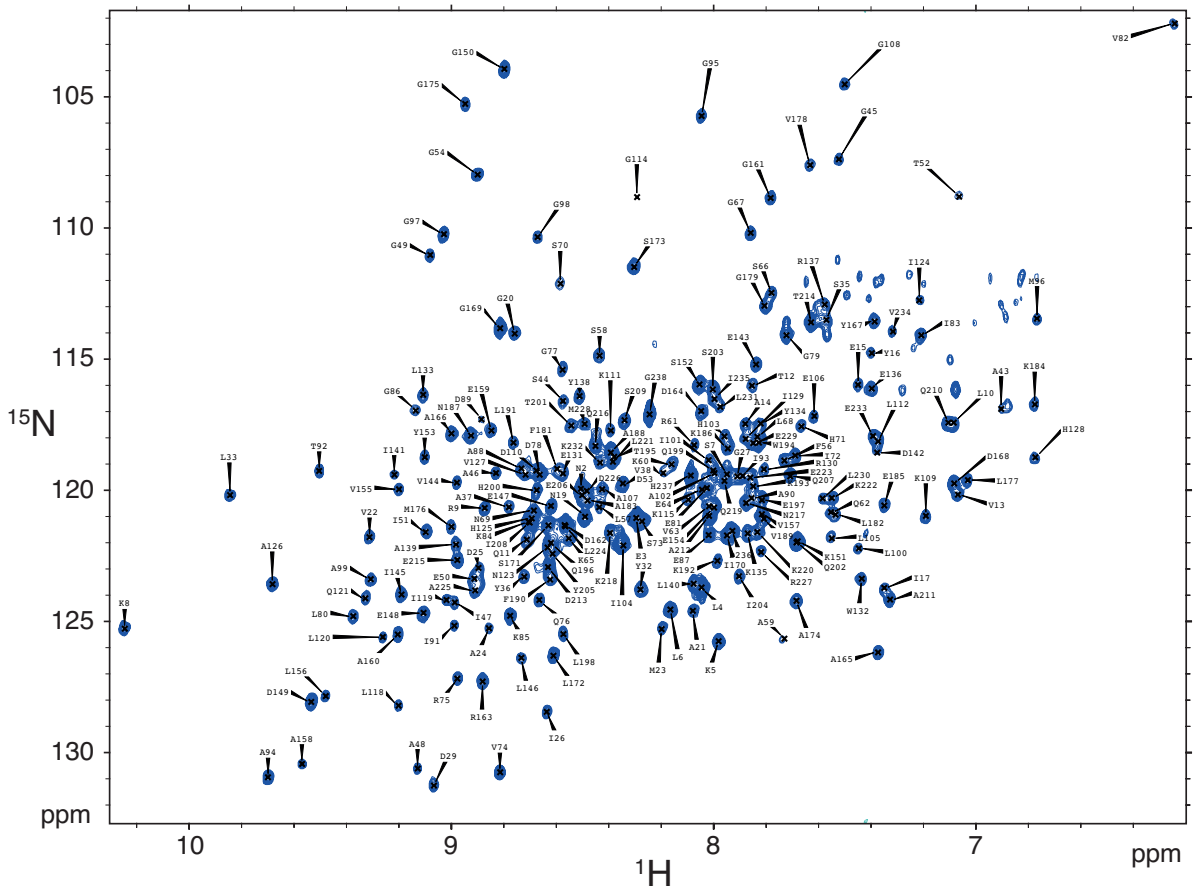

Figure 2. Two-dimensional ^{1}H-^{15}N TROSY spectrum of TrmK measured at 950 MHz and 15 °C on a protein sample uniformly deuterated and ^{15}N/^{13}C-labeled. Resonance assignments are indicated and reported in BMRB accession number 26744.

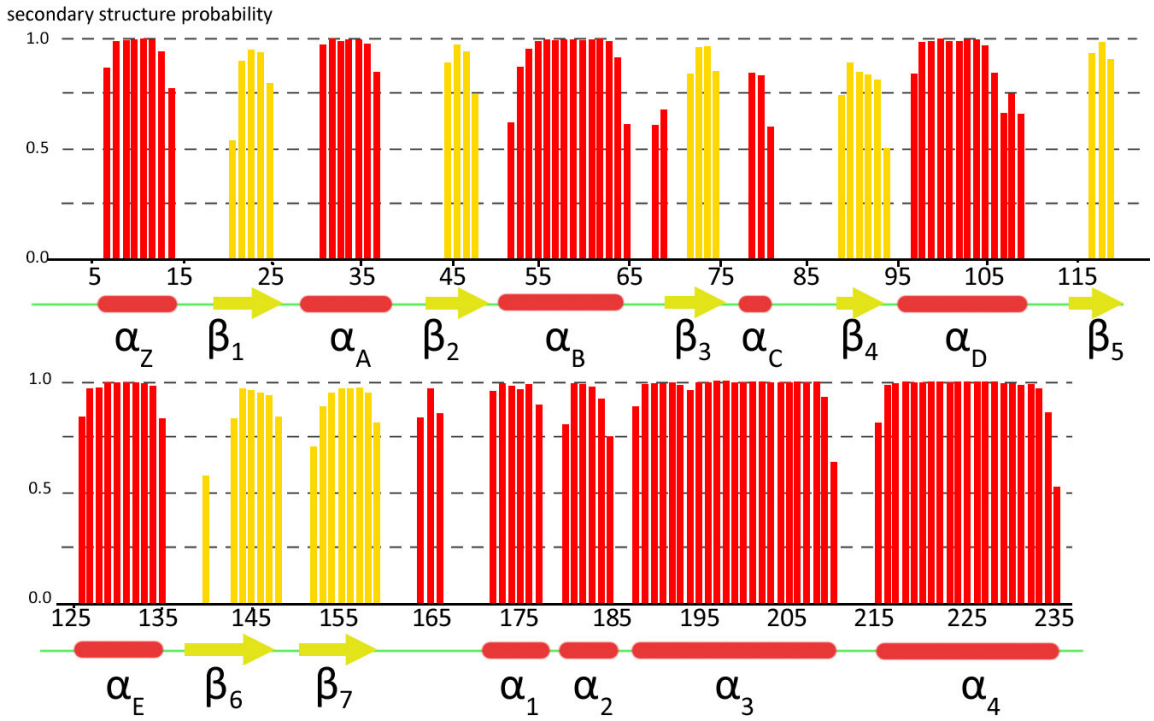

Figure 3. Prediction of TrmK secondary structure based on its backbone NMR chemical shifts using TALOS+ (Shen *et al.* 2009). The secondary structure prediction obtained with TALOS+ is shown as red bars for α-helices and yellow ones for β-strands, the height of the bars represent the probability of the secondary structure assigned by the software. Secondary structure of the TrmK X-ray structure is reported under the graph with yellow arrows for β-strands and red cylinders for α-helices.